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Review

Extracellular Nucleic Acids and their Potential as Diagnostic, Prognostic and Predictive Biomarkers

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Abstract. *Extracellular nucleic acids (NAs), both DNA and mRNA, have been found to exist in many biological media, including serum, plasma, saliva, urine, semen, milk and bronchial lavage, as well as cell culture supernatants. Analysis of such NAs as potential diagnostic, prognostic or predictive biomarkers for cancer has indicated that, while these NAs are detected in both plasma and serum from both healthy individuals as well as those suffering from a broad range of cancer types, their overall concentrations in the circulation are generally higher in cancer than in normal conditions. Indeed, the detection of specific mRNAs (by RT-PCR/qPCR and, more recently, by microarrays) has been associated with the presence of cancer, supporting their potential as useful biomarkers. Furthermore, it has been proposed that these extracellular mRNAs are not inert, but may have functional relevance. Evidence suggests that such extracellular NAs also have potential as biomarkers for a range of other pathological conditions and in forensic science. In conclusion, it seems that the field of extracellular NAs has great potential to be exploited by the development of minimally-invasive diagnostic, prognostic and predictive assays for cancer if it is ensured that relevant and adequate controls are included in all studies.*

An Introduction to the Discovery of Extracellular Nucleic Acids

Extracellular DNA. The initial discovery of extracellular nucleic acids (NAs) dates back almost 90 years. In 1928, the transmission of pathogenicity from heat-killed infectious bacteria to viable non-pathogenic strains was reported (1). This transformation – through the transfer of DNA between bacterial strains – was later verified and shown to be an active process and not linked to cell lysis (2, 3). The concept of extracellular "messenger DNA" also finds support from plant studies, where such NAs have entered nuclei of cells, become integrated in endogenous DNA and are expressed (4).

The presence of cell-free circulating DNA in human plasma was initially reported in 1948 (5). The first association between circulating NAs and clinical conditions was described 40 years ago, when higher levels of circulating DNA were found in patients with systemic lupus erythematosus, compared to healthy individuals (6). Similarly, associations between elevated circulating DNA levels and other clinicopathological conditions, including pancreatitis, hepatitis, inflammatory bowel disease, glomerular nephritis, and rheumatoid arthritis (7) and, more recently, cancer (8) have been reported; with higher levels of circulating DNA associated with metastatic cancer compared to localised disease and reduced levels of DNA found following radiotherapy. Advancing on these findings of circulating DNA associated with cancer, in 1996 Nawroz *et al.* (9) reported microsatellite shifts, oncogene mutations and loss of heterozygosity in plasma and serum that matched those occurring in primary tumours.

Extracellular RNA. The presence of extracellular RNA in the medium of eukaryotic cell cultures has also been described. In 1978, Stroun *et al.* (10) reported the presence of an RNA form in a nucleoprotein complex spontaneously released from human blood lymphocyte and frog auricle cultured cell systems. The resulting isolated RNA was described as not being tRNA and being more highly methylated than ribosomal 28S, 18S and 4-5S RNA. More recently, using a fluorescence-based assay, the detection of cell-associated and cell-free (following 1.2 μm filtration) RNA in growth/culture medium from HeLa (human cervical carcinoma cell line) and A431 (human squamous cell line) cells, represented by 100-200 nucleotide long fragments, corresponding to 2.5-5S RNA, has been reported (11).

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Using RT-PCR techniques, we detected many gene transcripts – including *mdr-1, mrp-1, CK-19, HnRNP B1, GST-π, topoisomerase II, bcl-2* and *β-actin* – in medium conditioned by a broad range of healthy, proliferating, human cancer cell lines (12). At least some of the mRNAs analysed were apparently full-length - possibly particle-bound - transcripts and not all mRNAs found to be transcribed by a given cell line were detected in its conditioned medium, suggested a selective process of passing mRNA into the surrounding environment. As described in more detail below, as for DNA, a number of studies have also detected RNA presence in human plasma and serum.

Although extracellular NAs have now been detected in a range of biological matrices, including serum/plasma (see Table I), saliva (31, 32), urine (33), milk (34), bronchial lavage (35), as well as cell culture supernatants (10-12), how they are released from cells – whether that be by active secretion, as a result of apoptosis/necrosis, or some combination of these mechanisms – is not yet defined (36). The protection of extracellular RNA from degradation by RNases may be due to it being packaged into apoptotic bodies (37), integrated within nucleoprotein complexes (38), with phospholipids (39, 40) and proteins (41). For example, while filtration through a 0.22 μm filter of plasma from hepatocellular carcinoma (HCC) patients and healthy individuals did not result in a reduction in extractable DNA, analysis of GAPDH mRNA levels indicated that filtration reduced mRNA concentrations by up to 15-fold, suggesting this mRNA to be associated with circulating particles (23). Likewise, in our study of cancer cell line conditioned medium (CM), we found that some transcripts were detectable following 0.45 μm filtration, but were no longer amplifiable following filtration of the same CM through a 0.22 μm filter (12).

Extracellular Cancer Biomarkers

Cancer is frequently diagnosed as a result of the chance finding of a lump which is subsequently biopsied for confirmation and sub-classification, based on a limited number of gross pathological features and immunochemical markers. The procedures available for procuring specimens for diagnosis are invasive and the methods for diagnosis are often relatively crude and subjective. Currently these are the only means available to surgeons/clinicians/oncologists to assist in the decision on the patient's course of treatment - whether that be nothing but on-going observation, surgery alone or followed (or preceded) by chemotherapy/radiotherapy/hormonal therapy, palliative care alone, *etc.* Years of experience have shown, however, that patients diagnosed with the same stage of cancer by conventional clinical and histopathological criteria may have a completely different course of disease. As cancer is fundamentally a malfunction of gene expression that gives rise to malignant growth, the most direct classification approach would involve analysis of gene expression patterns, as these molecular differences may be crucial. Unfortunately, clinical and pathological assessment of the tumour cannot distinguish between such morphologically similar, but possibly molecularly different, tumours. Furthermore, this approach allows analysis at only one particular time point in the existence of a tumour and in one location in the body. Minimally-invasive molecular methods are needed to enable earliest possible diagnosis and classification of cancer, to discern subtle differences that may be of importance for developing a better understanding of the tumour/its likelihood of spreading to other sites, and for assisting in selecting the best possible treatment for the individual cancer patient. Effective, clinically useful, cancer biomarkers should be accurately detectable in a readily accessible body fluid, such as serum, saliva or urine, permitting minimally-invasive procedures and ongoing/sequential monitoring of the course of the disease (*e.g.* progression, response to therapy) over time.

Extracellular biomarkers currently assessed in the clinic. The main extracellular "cancer biomarkers" routinely assessed in the clinic are oncofetal proteins in blood specimens. These have been identified in embryo/fetus, are diminished to low levels in the adult, but reappear in tumours. These oncoproteins (www.tc-cancer.com/tumormarkers.html) include carcinoembryonic antigen (CEA) (www.medicinenet.com/carcino-embryonic_antigen/article.html), the first oncofetal antigen to be exploited clinically. However, although initially identified as a marker for colon cancer, an abnormal CEA blood level is neither specific for colon cancer nor for malignancy in general. In addition to occurring in association with other cancer types (including pancreatic, stomach, lung, breast and testicular), elevated CEA levels are detected in benign conditions, including cirrhosis, inflammatory bowel disorder, chronic lung disease and pancreatitis. CEA, however, is also elevated in 19% of smokers with no identified disease and in 3% of healthy non-smokers. Alpha-fetoprotein (AFP) (www.medicinenet.com/alpha-fetoprotein_blood_test/article.html; www.acor.org/cnet/62840.html), while an associated marker for HCC and germ cell carcinoma, colonic, bronchogenic and gastric cancer, its blood levels are also elevated in benign liver disease (including hepatitis and cirrhosis) and in normal pregnancy. Indeed, it was recently shown that the sensitivity of AFP for HCC is approximately 60% *i.e.* an elevated AFP blood test result is found in about 60% of HCC patients; that leaves 40% of patients with HCC who have normal AFP levels. While elevated CA125 (www.tc-cancer.com/tumormarkers.html) levels are associated with endometrial, pancreatic, lung, ovarian, breast and colon

cancers, these also occur in menstruation, pregnancy, endometriosis and other conditions. Prostate-specific antigen (PSA) [cgap-mf.nih.gov/ProstateExample/ProstateMicroReviews/Diag; (42)], a protein normally present at low levels in blood of adult men, is probably the most commonly tested tumour marker. While elevated PSA levels are associated with the presence of prostate cancer and a PSA test combined with rectal examination has been shown to be superior to rectal examination alone for prostate cancer detection, it must be remembered that PSA is prostate-specific, not cancer-specific. Indeed, in a large-scale study of PSA screening for prostate cancer (43), it was concluded that, contrary to current clinical practice, there is no definitive "cut-off point" PSA level associated with cancer. Some prostate glands normally produce more PSA than others. In addition to this, a number of conditions, including prostatitis (prostate inflammation) and benign prostatic hypertrophy (prostate enlargement) result in raised PSA blood levels. It is obvious, therefore, that the detection of specific biomarkers in plasma and/or serum may greatly assist in the detection and, thus, treatment of these diseases.

Extracellular mRNA cancer biomarkers. Increased levels of circulating DNA in cancer patients, compared to healthy individuals, were initially reported in 1987. However, as studies of circulating DNAs have been reviewed by others (for example, see 44-45), the main focus of this review is on circulating RNAs.

The first study associating circulating RNA, as RNA-proteolipid complexes, in serum as potential tumour markers was reported by Wieczorek *et al.* in 1987 (46). This study reported an association between the presence of RNA-proteolipid complexes and tumour mass/response to therapy. These complexes disappeared approximately 48 hours after tumour removal and were undetected in benign disorders. Despite the instability of mRNA and the presence of RNases in serum, with increased levels of RNases reported in sera from cancer patients (47, 48), over the past 7 years a number of studies (summarised in Table I) have indicated that it is possible to detect extracellular mRNA in the serum and/or plasma of patients with melanoma, lung, breast, prostate, oral, bladder, liver, thyroid and colorectal cancers and lymphomas, as well as in normal plasma and/or serum (13-30). These analyses generally involved studies of mRNAs on a one-at-a-time basis, often by RT-PCR/qPCR.

Recently, Li *et al.* (49) reported promising results from analysis of sera mRNA from patients presenting with oral squamous cell carcinomas compared to normal serum controls, using an Affymetrix array (U133A) containing approx. 22215 probesets and representing approximately 19000 genes, indicating the potential of this approach. In the first whole genome expression microarray analysis (approximately 54675 transcripts/variants), aimed to establish the feasibility of analysing global gene expression profiles in human serum, including RNA isolated from serum from breast cancer patients and healthy volunteers, we detected more than 6000 extracellular transcripts (manuscript in prep.).

In addition to diagnostic and prognostic potential, a very limited number of studies have attempted to investigate the feasibility of identifying mRNAs/panels of mRNAs that are potentially predictive of response to therapy. These included studies of *Her-2* and *hnRNP-B1* mRNAs in lung cancer (19) and *PSMA* and *CEA* mRNAs in prostate cancer (29). Although both studies reported the number of mRNA-positive cases to be lower post-therapy when compared to pre-therapy, it must be considered that the patient groups analysed post-therapy were not the same as those analysed pre-therapy in either study, limiting conclusions that may be drawn from this data.

Have Extracellular Nucleic Acids a Biological Role?

A limited number of studies to date have suggested that circulating NAs may not be inert, but may have biological relevance. Forty years ago it was established that, through what seems to be an active process (as uptake is decreased when metabolic activity of cells is lowered), RNAs are readily taken up by ascites tumour cells (50, 51). More recently, studying co-cultured cells that were previously incubated with or without tantalum particles, intact labelled RNA – but not DNA – was found to transfer into the non-labelled recipient cells, from the labelled donor cells (52).

Biological relevance of mRNAs transferred to/uptaken by cells has been suggested in both plant and animal cells. For example, Huang *et al.* (53) reported that mRNA encoding the *Arabidopsis* gene, FT, migrates from the leaf to the shoot apex where it induces flowering, while Anker *et al.* (54) reported transformation of NIH-3T3 murine cells following exposure to medium conditioned by SW480 colon cancer cells. Indeed, based on studies in rat models where haematogenous dissemination was found to be more closely associated with circulating NAs than with circulating tumour cells, Garcio-Olmo *et al.* (55-60) proposed the "Genometastasis hypothesis" suggesting that metastases result from transformation of susceptible cells [possibly stem cells (61)] by circulating NAs.

Other Clinical Conditions

Assessment of circulating NAs has also shown diagnostic and/or prognostic potential in pathological conditions other than cancer. Within an hour of injury/trauma, plasma DNA levels have been reported to be elevated, with the concentration of circulating DNA associated with the severity of injury (62). β-globin DNA levels have been found

Table I. *Circulating mRNAs in serum/plasma.*

mRNA(s) studied	Cancer type	No. of positive cases / Total number		Reference
Tyrosinase[1]	malignant melanoma	4/6		(13)
	normal	0/20		
		Serum	*Plasma*	
Tyrosinase[1,2]	malignant melanoma	3/10	5/10	(14)
PBDG		7/10	8/10	
gp100		0/10	0/10	
MART-1		0/10	0/10	
5T4[1]	breast cancer	2/5		(15)
	NSCLC	6/14		
	normal	3/25		
		hRT	*hTERT*	
Telomerase (*hRT*)[1]	breast cancer	5/18	4/16	(16)
& *hTERT*	benign breast disease	0/2	0/2	
	normal	0/21	0/21	
Tyrosine	melanoma	24/119		(17)
MAGE-3		10/119		
MUC-18		41/119		
P97		19/119		
hTERT	follicular lymphoma	detected in all, but significantly higher in tumours		(18)
	colorectal cancer			
	normal			
CK-19[1]	lung	15/18		(19)
PGP 9.5		1/18		
Her2/neu		7/18		
hnRNP-B1		14/18		
TTF-1		0/18		
MAGE-2		0/18		
Mammaglobin[2]	breast	6/10		(20)
	normal	6/16		
		Mg	*CK-19*	
Mammaglobin (Mg)[2]	breast	27/45	22/45	(21)
CK-19	normal	3/25	5/25	
		CEA	*CK-19*	
CEA	colorectal	17/53	39/53	(22)
& *CK-19*	normal	1/25	5/25	
GAPDH[2]	HCC	detected in all cases; levels detected reduced with filtration & ultracentrifugation		(23)
	normal			
hTERT	HCC	89.7%		(24)
	liver cirrhosis	70.0%		
	chronic hepatitis	41.7%		
hTERT	colorectal	detected in both, but significantly higher in cancer		(25)
	normal			

Table I. *continued*

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mRNA(s) studied	Cancer type	No. of positive cases / Total number		Reference
		hRT	*hTERT*	
hRT &	breast	23/25	12/25	(26)
hTERT	melanoma	24/29	17/29	
	thyroid	4/4	4/4	
	normal	3/7	0/7	
β-catenin	colorectal cancer	58/58		(27)
	colorectal adenoma	49/49		
	normal	36/43		
hTERT	HCC	levels detected		(28)
	liver cirrhosis	significantly higher in		
	chronic hepatitis	HCC than in other cases		
		PSMA	*CEA*	
PSMA &	prostate cancer pre-therapy	4/12	1/12	(29-30)
CEA	prostate cancer during therapy	3/19	3/19	
	normal	0/9	0	

[1]Serum analysed; [2]plasma analysed; normal: blood specimens were taken from volunteers who did not have cancer; CEA: carcinoembryonic antigen; HCC: hepatocellular carcinoma; PSMA: prostate-specific membrane antigen.

to be elevated in stroke victims (63), with plasma β-globin DNA alone reported to be a better discriminator of haemorrhagic from non-haemorrhagic stroke than serum S100 protein, especially within 6 hours of symptom onset (64). Indeed, elevated circulating levels of DNA and RNA associated with stroke, trauma and acute coronary syndrome are generally higher in patients with a high risk of death and have been found to be prognostic in chest pain patients (65). Circulating *rhodopsin* mRNA levels are higher than normal in patients with diabetes retinopathy (66). Increased rhodopsin mRNA levels in diabetic patients with retinopathy were also reported by Butt *et al.* (67), who additionally described significantly elevated levels of *nephrin* mRNA levels in normoalbuminuric diabetics compared to healthy controls.

Serum or Plasma for Analysis of Cell-free Nucleic Acids?

To date, many more studies have been performed on circulating DNA, rather than RNA. Some studies have mainly focussed on analysis of plasma, while others have isolated NAs from serum for analysis. However, it has yet to be established which medium is most suitable and relevant to study.

A limited number of studies have compared DNA levels in serum and plasma and have reported levels to generally be higher in serum. For example, based on analysis of serum and plasma from healthy individuals, Cox and Gocken (68) reported 35 ng/ml DNA in plasma compared to 318 ng/ml in serum. Similarly, Gautschi *et al.* (69) reported DNA levels in healthy subjects as 1.8 ng/ml in plasma and 12.6 ng/ml in serum, with 3.7 ng/ml and 39.6 ng/ml, respectively, in plasma and serum from non-small cell lung cancer patients. Likewise, in their studies of plasma and serum, Thijssen *et al.* (70) detected 4.8 ng/ml DNA and 12.9 ng/ml DNA in plasma and serum, respectively, from healthy individuals, with 10.6 ng/ml and 47.6 ng/ml detected when analysing matched specimens from those with colorectal cancers metastasised to the liver.

Such extensive analysis has not yet been undertaken for RNA, with most quantitative studies so far focussing on plasma analysis. The reported concentrations of RNA include 1-10 ng/ml (71) and 142 ng/ml (72) in plasma from healthy individuals. In studies of lung cancer compared to normal plasma, Laktionov *et al.* (73) detected 920 ng/ml and 250 ng/ml in cancer and normal cases, respectively. In a similar study, 710 ng/ml RNA was found in normal plasma and 860 ng/ml in lung cancer plasma (74). These are in comparison to 20-70 μg/ml RNA in human milk (34) and 24-140 ng/ml RNA in urine of healthy individuals (33).

While direct comparisons of RNA in plasma and serum from the same specimens are not available, overall the levels of free RNA (like DNA) circulating in blood from cancer patients is apparently higher than that in healthy individuals, supporting its potential role as a useful cancer biomarker.

Forensic Medicine

Analyse of extracellular mRNA profiling in forensic science has also been investigated and, again, although studies have been limited so far, results suggest that this approach has potential. Using standard RT-PCR and gel electrophoresis, Juusola and Ballantyne (75) reported that mRNAs specific for saliva and semen can be detected from stains as old as 10 weeks. Advancing on this preliminary work, in a proof-of-concept study, where *β-spectrin* and *porphobilinogen* were selected as indicators of blood; *statherin* and *histatin 3* as indicators of saliva; *protamine 1* and *protamine 2* as indicators of semen; and *β-defensin 1* and *mucin 4* as indicators of vaginal secretions, a multiplex RT-PCR assay was developed that could detect these four body fluids as single or mixed stains (76). The potential usefulness of this approach was further supported by qPCR analysis indicating that neither the small amounts of trace specimens nor long storage times at ambient temperatures (which realistically may occur prior to analysis in a forensic lab.) inhibited successful qPCR analysis (77).

Extracellular Nucleic Acid Analysis: A Note of Caution

It has recently been suggested (78) that expression microarray and qPCR analysis of saliva specimens might be detecting genomic DNA, rather than mRNA, as previously suggested (31). This assumption was based on the analysis of "no-RT" (*i.e.* no reverse transcriptase enzyme included in cDNA reaction: $RT^{(-)}$) and "+RT" ($RT^{(+)}$) conditions yielding similar amounts of PCR product; microarrays signals, being unaffected by RNase-treatment; the absence of RT-PCR products following DNase-treatment; and the absence of RNA-specific RT-PCR products. These are very important points which should be considered and results from studies performed without relevant controls must be viewed with caution.

While the following does not necessarily relate directly to saliva, it is important to also consider that analyses performed by others, where relevant controls have been included, support the presence of circulating mRNA in serum. For example, while our microarray results (manuscript in preparation) cannot definitively rule out the presence of DNA encoding for any of the approximately 55,000 transcripts analysed if – as suggested by Kumar *et al.* (78) – the amplification and labelling methods routinely used prior to applying cRNA onto Affymetrix microarrays may lead to some false-positives due to DNA pseudogene contamination, our qPCR analysis on aliquots of the same RNA as used for microarrays, including both $RT^{(-)}$ and oligo $dT^{(-)}$ controls, resulted in no detectable products for any of the 22 serum specimens and 14 gene products analysed, even after 40 cycles of amplification. This observation supports the assumption that the NAs that we detected in our serum studies are of RNA, not genomic DNA, origin. Similarly, in their assessment of the usefulness of extracellular mRNA analysis in forensic science, Juusola and Ballantyne (76) eliminated the possibility that their analysis was of genomic DNA in error (and not mRNA, as described) by treating RNA with DNase enzymes, including $RT^{(-)}$ controls, and designing exon-spanning primers that would either not amplify DNA or would result in a much larger product than that from cDNA. Similar considerations were made in the study reported by Nussbaumer *et al.* (77).

Conclusion

Although the analysis of extracellular NAs is still in its infancy, progress in this field to date suggests that NAs can be detected in many body fluids, including serum, plasma, saliva, urine, milk, semen, vaginal secretions and bronchial lavage and may be potential biomarkers for a range of pathological conditions including cancer, diabetes, stroke, trauma and acute coronary syndrome. Furthermore, the indications that such extracellular mRNAs may have a biological role and may be involved in transforming cells, suggests that in cancer these NAs may have potential as diagnostic, prognostic and predictive biomarkers, and may also be considered as therapeutic targets. Further, well-controlled analysis of extracellular NAs in studies of larger cohorts of patients and healthy volunteers will help us to determine how best to exploit these exciting findings in the interest of cancer patients.

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